96-well Bisulfite Library Construction for Illumina Sequencing

I. Purpose

To provide specific guidelines for 96-well automated Library Construction and Bisulfite Conversion for Illumina Paired-End Sequencing using NEB premix library construction reagents, Zymo EZ-96 DNA Methylation Gold MagPrep and KAPA Biosystems HiFi Hot Start Uracil Ready PCR enrichment kit.

II. Scope

All procedures are applicable to the BCGSC Library Core and Library TechD groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Assurance Management to audit this procedure for compliance and maintain control of this procedure.

V. References

Reference Title	Reference Number
Sample Preparation for Paired-End Sample Prep Kit from Illumina EZ-96 DNA Methylation-Gold MagPrep Version 1.0.4 KAPA HiFi Hotstart Uracil+ Ready Mix PCR Kit	Version 1.1 (from Prep Kit) Cat D5042 TDS KR0413 v2.13

VI. Related Documents

Document Title	Document Number
96-well DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V	LIBPR.0108
Operation of the Covaris LE220	LIBPR.0097
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017

Document Title	Document Number
Operation and Maintenance of the Caliper Labchip GX for DNA samples using the High Sensitivity Assay	LIBPR.0051
Quantifying DNA samples using the Qubit Fluorometer	LIBPR.0030
Normalization of Nucleic Acid Concentration using the JANUS Automated Workstation	LIBPR.0113
Span-8 Pooling of DNA Samples	LIBPR.0093

VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material safety data sheet (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
NEB End Repair Mix	NEB	E6875B-GSC		✓
NEB dA-Tail Mix	NEB	E6876B-GSC		✓
NEB Ligation Mix	NEB	E6877B-GSC		✓
KAPA HiFi Uracil+ Ready Mix PCR Kit	KAPA	KM2801		✓
Alpaqua 96S Super Magnet Plate	E & K Scientific	A001322		✓
Alpaqua MAGNUM FLX, 96M Ex (Large volume)	E & K Scientific	A000400		✓
Zymo EZ-96 DNA Methylation-Gold MagPrep	Cedarlane	D5042		✓
CT conversion reagent (10 reactions) for added flexibility	Cedarlane	D5001-1	✓	
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53	✓	
Soft Touch (pink) gloves – various sizes	Ultident	296359683		✓
Ice bucket	Fisher	11-676-36		✓
Wet ice	In house	N/A	N/A	N/A
Covaris LE220 with WCS and Chiller	Covaris	LE220		✓
DNA *AWAY*	Molecular BioProducts	21-236-28		✓
AB1000 96-well 200µl PCR plate	Fisher	AB1000		✓
Gilson P2 pipetman	Mandel	GF-44801	✓	
Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		
Gilson P200 pipetman	Mandel	GF-23601	✓	
Gilson P1000 pipetman	Mandel	GF-23602	✓	
Diamond Filter tips DFL10	Mandel Scientific	GF -F171203	✓	
Diamond Filter tips DF30	Mandel Scientific	GF-F171303	✓	
Diamond Filter tips DF200	Mandel Scientific	GF-F171503	✓	
Diamond Filter tips DF1000	Mandel Scientific	GF-F171703		✓
Galaxy mini-centrifuge	VWR	37000-700		✓
VX-100 Vortex Mixer	Rose Scientific	S-0100		✓
Black ink permanent marker pen	VWR	52877-310		✓
Clear Tape Sealer	Qiagen	19570	✓	

Aluminum Foils seals	VWR	60941-126	✓	✓
Aluminum foil tape, 3"x 60 yds	Scotch/3M	34000740		✓
Eppendorf BenchTop Refrigerated Centrifuge 5810R	Eppendorf	5810 R		✓
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		
Small Autoclave waste bags 10"X15"	Fisher	01-826-4		
Anhydrous Ethyl Alcohol (100% Ethanol)	CommercialAlcohols	00023878	✓	
IKA Works Vortexer	Agilent	MS2S9-5065-4428		✓
Peltier Thermal Cycler	MJ Research	PTC-225		✓
Power Supply, LVC2kW, 48VDCV	Tyco Electronics	RM200HA100		✓
P165B Tips, sterile, 10 racks of 96/box	Ultident	24-FXF-180-LRS	✓	
P50 (Universal) Tips, Presterile with Barrier, 50 µL, 96/rack, 10racks/case, CS960	Beckman	CABKA21586	✓	
Plate, 96-Well reservoirs, diamond-bottom, Low-Profile	Seahorse	EK2036	✓	
Plate, 96-Well reservoirs, diamond-bottom, Deep-Profile	Seahorse	S30014		✓
Plate, 96-Well reservoirs, 450uL EtOH & Waste	PerkinElmer	20815114		✓
Plate, 96-Well 1.2mL Binding & Waste	Abgene	AB-1127		✓
Falcon tube for bead/binding buffer preparation		352070		✓
Biomek FX Liquid Handling System	Beckman	Biomek FX		✓
Eppendorf Benchtop Centrifuge	Eppendorf	5810 R		✓
70% Ethanol	In house	N/A	N/A	N/A
Qiagen Buffer EB – 250 mL	Qiagen	19086	✓	
10 X TBE - 10 L	Invitrogen	15581-028	✓	
1 X TBE	In House	N/A	N/A	N/A
UltraPure Distilled Water	Invitrogen	10977-023		✓
AMPure XP Paramagnetic Beads	Agencourt	000132		✓
Unmethylated Lambda DNA	Promega	D1521		
PE primer 1.0	IDT	N/A		✓
PE indexed primers	IDT	N/A		✓
Methylated PE adapters	IDT	N/A	✓	
Nuclease Free 2.0 mL eppendorf tube	Ambion	12400		✓
5 mL Screw Cap tubes	Ultident	SCT-5ML-S		✓
Aline PCR Clean DX beads	Aline	C-1003		✓

These sequences are for internal use only:

Methylated PE adapters (for ordering):
A/iMe-dC/A/iMe-dC/T/iMe-dC/TTT/iMe-dC//iMe-dC//iMe-dC/TA/iMe-dC/A/iMe-dC/GA/iMe-dC/G/iMe-dC/T/iMedC/TT/iMe-dC//iMe-dC/GAT/iMe-dC/*T
***** Phosphorothioate Bond

5Phos/GAT/iMe-dC/GGAAGAG/iMe-dC/GGTT/iMe-dC/AG/iMe-dC/AGGAATG/iMe-dC//iMe-dC/GAG

Methylated PE adapters (in plain text):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3' GAGCCGTAAGGACGACTTGGCGAGAAGGCTAG

PE PCR Primers

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5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
5'CAAGCAGAAGACGGCATACGAGATNNNNNNCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT
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IX. Introduction and Guidelines

1. General Guidelines

1.1. Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment. Treat everything with clean PCR techniques.
1.2. Wipe down the assigned workstation, pipetman, tip boxes and small equipment with DNA *AWAY*. Ensure you have a clean working surface before you start.
1.3. Pre-PCR and Post-PCR work should be performed on the 5th Floor and 6th floor respectively.
1.4. Acronyms: NA stands for Not Applicable. Pre-LC refers to Pre-Library Construction. Post-LC refers to Post-Library Construction. BC refers to Bead Clean.
1.5. Discuss with the APC/PC/designated trainer the results of every QC step. Report and record equipment failures and/or malfunctions and variations in reaction well volumes.
1.6. Aline beads may be used interchangeably with Ampure XP beads but the two bead types should not be mixed.

2. General Plate Guidelines

2.1 Up to 4 plates can be processed at a time for library construction by one technologist using Biomek FX. Bisulfite conversion cleanup can be processed using one plate at a time on Biomek FX.
2.2 To avoid cross-well contamination, reaction plates should never be vortexed and plate seals should never be re-used. Use Biomek FX for mixing.
2.3 Use only VWR foil seals for short term storage and tetrad incubations/PCR, and 3M aluminum foil seal for long term storage.
2.4 Quick spin the plate(s) at 4ºC for 1 minute at 2000 g after incubation.
2.5 Sample plates can be stored at -20ºC overnight after every step except post Adenylation. Adenylation and Ligation must be performed on the same day.

3. Positive and Negative Controls

3.1. One microgram of HL60 genomic DNA is used as a positive control for this pipeline. In addition, 10ng of unmethylated Lambda DNA will be spiked into control and collaborator's gDNA samples to measure bisulfite conversion efficiency. The yield of library products constructed from positive controls is expected to differ from those of collaborators'

samples. However, the yield should not differ significantly from that of previously constructed positive controls.

3.2. Qiagen Elution Buffer is used as a negative control library construction. This control will measure any background products that result from the library construction process. Qiagen Elution buffer is also used as a negative control for bisulfite conversion and ultrapure water is used as a negative control for PCR enrichment.

4. General Brew Preparation Guidelines

4.1. Double check the QA release and expiry date of each reagent.
4.2. Thaw required reagents and premixed brews and place them on ice. Enzymes should be left in the freezer until ready to use.
4.3. Reagents and enzymes should be well mixed, the former by pulse-vortexing and the latter by gentle tapping of the tube or inversions. Treat premixed brews as enzymes. After mixing, quick spin in a mini-centrifuge.
4.4. Once prepared, all brews should be well mixed by gentle, repeated pulse-vortexing to ensure equal distribution of all components and thus uniformity of enzymatic reactions across a plate. The Ligation brew is particularly viscous.
4.5. All reactions require the preparation of a Brew Source Plate. The Biomek FX will be used to aliquot the brew from the Brew Source Plate into the reaction plates unless otherwise noted.
4.6. All brew calculators include excess volume to account for dead volume required by the Biomek FX in the Brew Source Plate and to account for pipetting loss.
4.7. Follow instructions in this SOP to determine the volume of premixed brew per well.

5. Biomek FX Handling Guidelines

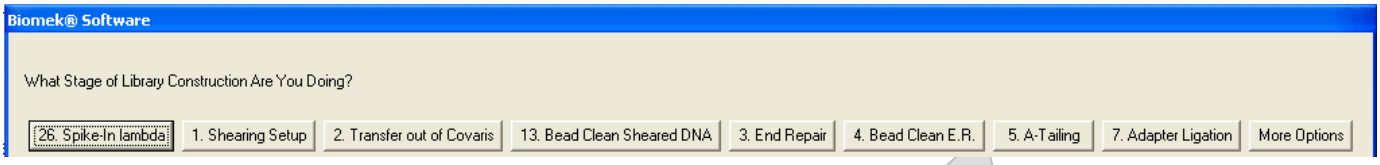
5.1. Home all axes before starting each Biomek run
5.2. Reaction brews vary in viscosity, selecting the correct Biomek technique is therefore essential to ensure accurate volume transfer.
5.3. The default dead volume required by the Biomek FX in each well of a Brew Source Plate/Indexing Primer plate is 5µL/well regardless of the number of plates being processed.
5.4. The dead volume required by the Biomek FX in the 96-well reservoir is 25mL.
5.5. Confirm the plate and tip box locations on the Biomek deck matches the software deck layout on the computer screen.
5.6. Ensure plate seals and tip box covers are removed before starting the Biomek program.

6. General note on Biomek programs

The following steps are generally followed:

A. Open project: LIBPR

B. Select and Run: LibraryConstruction
C. Select starting material type: Bisulfite
D. Select Library Construction step. An example is shown below:



The Biomek bead cleanup modules employed in this SOP are based on the following conditions:

Bead Binding Time (mins)	1st Magnet Clearing Time (mins)	2 X 70% EtOH Wash Vol (µL)	Ethanol Air-dry Time (mins)	Elution Volume (µL)	Elution time (mins)	2nd Magnet Clearing time (mins)
15	7	150	5	20-52	3	2

Note: Bead to reaction ratios are defined at each step

X. Procedure

1. Initial QC

1.1 For each gDNA 96 well stock plate, quantify according to the following SOP:

LIBPR_WORKINST.0108 96-well DNA Quantification using Quant-iT and VICTOR3

1.2 The recommended input is 1ug of high-quality genomic DNA.

2. Sample Normalization on the JANUS Automated Workstation

2.1 Normalize input as directed by your APC and according to the following SOP:

LIBPR.0113 Normalization of Nucleic Acid Concentration using the JANUS automated workstation

3. Lambda Spike-in and Shearing

3.1 Log into the following Biomek protocol to add 10ng of Lambda DNA to each well of the normalized gDNA plate.

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Spike-in Lambda**

3.2 To transfer DNA/cDNA into the Covaris plate, log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Shearing Setup**

3.3 Refer to the following SOP for shearing conditions:

LIBPR.0097 Operation of the Covaris LE220

Program: Plate_100sec_Bisulfite.elproc

Make sure that you have performed the shearing twice with a spin in between according to the SOP above.

4. Agilent HS DNA QC after shearing – Spot Check

4.1 For each 96 well plate of sheared samples, use 1 µL from 11 random samples (ensure that at least one of these samples is a positive control) to spot check on a High Sensitivity DNA Agilent Assay. For a clinical plate, QC every sample.

4.2 Dilute selected samples 6X prior to QC on HSDNA chip.

4.3 Refer to the following SOP:

LIBPR.0017 Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples

4.4 See appendix Fig1 for the expected 300bp profile after shearing.

5. Transferring DNA out of Covaris Plate

5.1.To transfer sheared gDNA from Covaris plate to reaction plate (ie. AB1000 plate), log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Transfer out of Covaris**

6. Post-shearing cleanup

6.1. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Bead Clean Sheared DNA**

6.2. The Biomek will transfer beads at a 1:1 Bead:Sample ratio to ensure stringent removal of small products.

7. End-Repair and Phosphorylation Reaction

7.1. End Repair Premix must be thawed on ice. If required, place tube at room temperature in the Laminar flowhood for 10 minutes to allow the brew to completely thaw. Mix reaction thoroughly by tapping the tube and/or 10X inversions, then quick spin before aliquoting brew into an AB1000 plate or equivalent. Note that aliquot volumes include 5µL of dead volume.

	End Repair Premix (µL) per well
Aliquot: 1 plate	28.5
Aliquot: 2 plates	52
Aliquot : 3 plates	75.5
Aliquot : 4 plates	99
Volume per reaction	**23.5**
Total Reaction Volume	**58.5**

7.2. Log into the following Biomek FX Program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**End Repair**

7.3. The brew plate is the "Source" and the DNA plate(s) is the "Dest." Seal the plates and quick spin at 4ºC for 1 minute once the Biomek protocol is finished. Inspect the reaction plates for any variations in volume. The total reaction volume is 58.5µL.

7.4. Incubate End-Repair reaction plate(s) at 20ºC for 30 minutes. Select 'Plate', enter '58' for reaction volume and select 'Y' for heated lid.

Tetrad Program: Run > LIBCOR > ER

7.5. After the 30 minute incubation, store plate at -20ºC or proceed to Bead Clean End-Repaired DNA. Quick spin the plate before loading the plate on the Biomek.

8. Bead Clean End-Repaired DNA

8.1. Log into the following Biomek FX Program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Bead Clean ER**

8.2. The Biomek will transfer beads at a 1:1 Bead:Sample ratio to ensure stringent removal of small products.

8.3. Check wells for bubbles after the final EB addition. If required, cover the plate and quick spin to remove bubbles. The Biomek program will prompt the user to quick spin plate if bubbles are present at this step.

8.4. Note that end-repaired product can be stored at -20°C after the bead cleanup.

9. Addition of an 'A' Base (A-Tailing) Reaction

9.1. Adenylation and ligation must occur on the same day. Do not proceed with Adenylation unless you have time to process ligation and the first bead clean post ligation.

9.2. A-Tailing Premix must be thawed on ice, and then mixed with gently flicking prior to dispensing brew into the brew plate using volumes described below. Note that aliquot volumes include 5µL of dead volume.

	A-Tailing Premix (µL) per well
Aliquot: 1 plate	25
Aliquot: 2 plates	45
Aliquot : 3 plates	65
Aliquot : 4 plates	85
Volume per reaction	**20**
Total Reaction Volume	**50**

9.3. Log into the following Biomek FX Program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**A-Tailing**

9.4. The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

9.5. Incubate A-Tailing reaction plate(s) at 37ºC for 30 minutes; 70ºC for 5 minutes; 4ºC for 5 minutes; 4ºC hold. Enter '50' for reaction volume and select 'Y' for heated lid.

Tetrad Program: Run > LIBCOR > ATAIL

9.6. During the incubation prepare ligation brew for in-tandem ligation. **Note: do not bead clean adenylated library.**

9.7. After the incubation, immediately proceed to Adapter Ligation. Quick spin your plate(s) and store on ice until loading the plate on the Biomek.

10. Adapter Ligation

10.1. Prepare pipeline-specific adapter ligation brew as described below:

10.2. Methylated PE Ligation Reaction

Solution	Volume (µL) per well
Adenylated DNA	50
2X Premix Ligation Brew	21
10uM Methylated PE adapter	4
Total Reaction Volume	**75**

Methylated_ Ligation_Brew_40pmol (25 uL)

10.3. Generate the Ligaton Brew LIMS calculator:

LIMS: Mix Standard Solution > Methylated_Ligation_Brew_40pmol > Save Standard Solution

10.4. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator.

10.5. To minimize adapter-adapter ligation, prepare brew on ice immediately prior to Biomek transfer of template to the ligation brew.

10.5.1. Prepare the Ligation brew in an appropriate sized tube on ice according to the chemistry calculator.
10.5.2. Immediately after the brew is prepared, dispense 30µL (for one plate) of brew to each well using a Distriman.
10.5.3. Cover the brew source plate with plate seal and quick spin at 4ºC for 1 minute.
10.5.4. Keep plates on ice but *proceed quickly* to the next step.

10.6. Log into the following Biomek FX Program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Adapter Ligation**

10.7. The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

10.8. Incubate Ligation reaction plate(s) at 20ºC for 15 minutes. Enter '75' for reaction volume and select 'Y' for heated lid.

Tetrad Program: Run > LIBCOR > LIGATION

10.9. While the ligation incubation is running, set up the Biomek for AMPure XP Magnetic bead clean up. **Bead clean up must occur immediately after the ligation reaction is completed.**

10.10. After the 15 minute incubation, quick spin the plate and store plate on ice. Proceed immediately to AMPure XP Magnetic Bead Clean Up after ligation.

11. AMPure XP Magnetic Bead Clean Up after Ligation

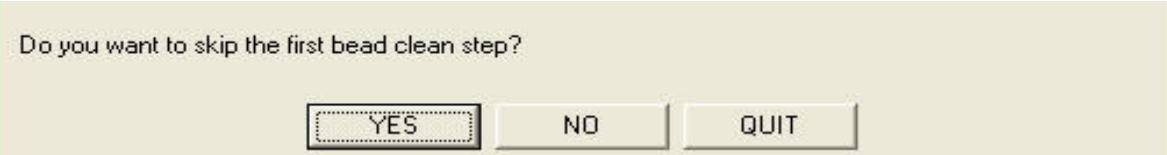
11.1. The input volume for this step is 75µL per well.

11.2. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Bead Clean Ligation(2X)**

11.3. Post-ligation bead cleanup is performed twice and a safe stopping point is after the first bead clean. A prompt will appear asking "Do you want to skip the first bead clean? Yes, No or Quit". If you want to proceed to the first bead clean and pause,

select "No". If you have already finished one round of bead clean and are continuing, select "Yes" (see Figure below).



11.4. Pour beads back to falcon tube and invert 10 times to mix the beads before the 2nd bead cleanup

11.5. Template can be stored at -20ºC or you may proceed immediately to bisulfite conversion.

12. Bisulfite Conversion

12.1. The Zymo Gold MagPrep kit is optimized for 200-500 ng of template DNA for optimal conversion efficiency. The current library construction pipeline yields 400-500ng in 20µL post ligation given 1ug of high quality gDNA and stringent removal of small inserts.

12.2. Prepare the CT Conversion reagent as described below.

Solution	Volume (mL)
Dried CT Conversion Reagent	NA
Ultrapure water	9
M-Dissolving Buffer	0.5
M-Dilution Buffer	3

12.3. Mix prepared conversion reagent with frequent vortexing or shaking for 15 minutes prior to taking an aliquot.

12.4. Conversion reagent must be protected from light. Cover tubes with foil or use light blocking tubes if preparing aliquots.

12.5. Prepared CT conversion reagent should be used immediately after preparation. If it is not being used immediately store the reagent at -20ºC for up to one month.

12.6. Pre-warm conversion reagent to 37ºC if using a previously frozen aliquot of the reagent.

12.7. Aliquot 150µL of prepared Bisulfite Conversion Reagent to an AB1000 plate using a distrimann pipette. Cover plate with a foil seal to protect the reagent from light. Quick spin plate and look for variations in volume. Top up low wells if necessary.

12.8. Log into the following Biomek FX program to transfer 20µL of template to a new AB1000 plate. A maximum of 500ng may enter bisulfite conversion.

Biomek: Project > LIBPR> AB1000

12.9. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Bisulfite Conversion**

12.10. Remove foil cover immediately before starting the Biomek run.

12.11. The Biomek will transfer 130µL of CT conversion reagent to 20µL of template.

12.12. Cover the plate with foil seal immediately after the transfer. Cover the plate with a tetrad incubation pad and incubate at 98ºC for 10 minutes, 64ºC for 2.5 hours and 4ºC for up to 20 hours. Enter '100' for reaction volume (max allowable) and select 'Y' for heated lid.

Tetrad Program: Run > LIBCORE > BSCONVER

13. Bisulfite Conversion Clean Up

13.1. Add 288 mL of anhydrous Ethanol to 72 mL wash buffer concentrate prior to starting Bisulfite Conversion Clean up. Indicate on the bottle that Ethanol has been added and record the date.

13.2. Prepare M-Elution buffer by aliquoting 50µL of M-Elution buffer per active well of an AB1000 plate. Cover the plate will a foil seal, quick spin, and then load on a tetrad and incubate at 55ºC:

Tetrad Program: Run > LIBCOR > 55Hold

13.3. Set a Peltier heat block with the 1.2mL plate adapter to 55ºC.

13.4. Prepare Binding buffer by adding 10µL of MagBinding Beads to 600µL of M-Binding Buffer for each reaction as described below in an appropriate container for the number of reactions being processed. Mix well by inversion.

	MagBinding Beads (µL)	**M-Binding Buffer (mL)**	**Total volume (mL)**
Per Reaction	10	0.600	0.610
12 samples	132	7.92	8.05
1 plate	1056	63.4	64.4

13.5. Log into the following Biomek FX Program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Clean Up Conversion**

13.6. The Biomek will ask if you would like the FX to pre-dispense wash buffer. Select yes if you are processing a full plate. If not, dispense M-Wash Buffer (with Ethanol added) into the appropriate wells of a P450uL plate using a Distrimann as described below:

	M-Wash Buffer (µL/well)
Wash 1	480
Wash 2	480
Wash 3	250

13.7. Follow the prompts to complete the deck layout. There are two layers. This process requires Alpaqua MAGNUM FLX, 96M Ex magnet, alternate magnets are not compatible.

13.8. Pulse-vortex Bead/M-Binding buffer and dispense 610µL per well in use using a distrimann and load plate onto the appropriate ALP on the Biomek. Remove plate covers and press play after double-checking the deck layout.

13.9. Aliquot 240uL of desulphonization buffer to the appropriate wells of a P450uL plate.

13.10. Note that desulphonization must not exceed 20-25 minutes as per the manufacturer. The Biomek process including mixing, binding and clearing totals <20minutes. If the Biomek fails during this step you must intervene and process the bead clean manually to prevent damage to your sample. Note a process deviation if this does occur.

13.11. The total Biomek process will take about 1.5 hours. Samples will be transferred and bound to beads in buffer and then cleared and washed. Samples will then undergo desulphonization and two wash steps. Samples are dried prior to hot elution.

13.12. At the end of the process, cover your bisulfite-converted DNA plate with an appropriate plate cover. Samples can be immediately enriched or the template can be stored at -20ºC until processed.

14. Indexed PCR Amplfication

Note: Prior to transferring template to the brew plate, remove the tip corresponding to the PCR brew control well from the tip box to avoid carrying over elution buffer to the PCR brew control well.

14.1. Thaw the PE PCR primer 1.0 in the Tissue Culture Room laminar flow hood on the 5th floor, room 511 and immediately place on ice.

14.2. Thaw the Indexing Primer Plate in a working bench across from Biomek FX on the 5th floor, quick spin at 4ºC for 1 minute and immediately place on ice.

14.3. To keep track of freeze-thaw cycles, mark off the indexing primer plate each time the plate is thawed even if it is not used.

14.4. The maximum freeze-thaw cycles for the indexing primer plate are 5 times.

14.5. Ensure there is enough volume for the number of plates to be processed including the Biomek dead volume.

14.6. Methylated iPCR brew (minus the primers) must be aliquoted in the PCR Clean Room laminar flow hood on the 5th floor, room 510. Addition of PE PCR primer 1.0 to the brew must be made in the Tissue Culture Room laminar flow hood on the 5th floor, room 511. Addition of the Indexing Primer Plate to the reaction plate is made by the Biomek FX.

14.7. Prepare the Bisulfite iPCR brew as described below:

Solution	Volume (µL) per well
KAPA 2X HiFi Ura+ Ready mix	25
PE PCR primer 1.0 (25uM)	1
Indexed PCR primer plate (12.5µM)	2

Bisulfite_LibConst iPCR_Brew (26µL)

14.8. Generate the appropriate Ligation Brew LIMS calculator:

LIMS: Mix Standard Solution > **Bisulfite_LibConst_iPCR_Brew** > *follow the prompts* > Save Standard Solution

14.9. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator. The indexing primers will be added to the Brew Source Plate using the Biomek FX.

14.10. Carefully dispense 26µL per well of an AB1000 plate using a Distrimann pipette. The Biomek protocol will transfer template directly to the brew therefore no dead volume is required in the brew plate.

14.11. Log into the following Biomek FX program:

Biomek: Project > LibraryConstruction >RUN> Bisulfite >**Index PCR**

14.12. The Biomek program for iPCR setup is as follows:

14.12.1. Addition of index primers to the Brew Source Plate.
14.12.2. Addition of all of the template into the Brew/Index Plate

14.13. Add 22 µL of water to the PCR brew control well.

14.14. After Biomek program completion, seal the plate(s) with VWR foil seal and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

14.15. Load the plate on a tetrad and run the following program. Enter '50' for reaction volume and select 'Y' for heated lid.

Tetrad Program: Run > LIBCOR > BS-5X

PCR Parameters

- 98˚C 1 min
- 98˚C 15 sec
- 65˚C 30 sec
- 72˚C 30 sec

Total of 5 cycles

- 72˚C 5min

- 4˚C ∞

15. Post-LC Bead Clean (2x 1:1)

15.1. To purify the iPCR product, log into the following Biomek FX program:

Biomek: Project >LibraryConstruction >RUN> Bisulfite > **Bead Clean iPCR (2X)**

15.2. Post PCR cleanup is performed twice for all protocols and a safe stopping point is after the first bead clean if desired. A prompt will appear asking "Do you want to skip the first bead clean? Yes, No or Quit". If you want to proceed to the first bead clean and pause, select "No". If you have already finished one round of bead clean and are continuing, select "Yes." This is important as the volumes going into the first and second bead cleans are different.

15.3. Pour beads back to falcon tube and invert 10 times to mix the beads before the 2nd bead cleanup.

15.4. Proceed to preparing dilutions for QC or store final library product at -20ºC in the designated post-PCR freezer.

16. Preparation of Diluted Library QC Plate

16.1. Prepare a dilution QC plate using the following Biomek FX program:

Biomek: Project >LibraryConstruction >RUN> Bisulfite > **Dilute for QC**

16.2. The Biomek will transfer 2µL of fully constructed library into 18µL of Qiagen EB. This 10X dilution will be used first for Quant-iT (2µL) and the remaining 18µL of the 10X dilution will subsequently be used for Caliper QC.

16.3. For partial plates, samples can be quantified using HS dsDNA Qubit.

17. Quant-iT/Qubit QC on Samples for pooling

17.1. Refer to the following SOPs for setting up the QC plate prior to pooling:

LIBPR.0108 96-well DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V or LIBPR.0030 Quantifying DNA Samples using the Qubit Fluorometer

17.2. For Quant-iT, use the 10X dilution plate from the previous step or undiluted library as source plates for the QC.

17.3. Log into the following Biomek program:

Biomek: Project >LibraryConstruction >RUN> Bisulfite > **Quant-iT**

17.4. For Qubit, use the undiluted DNA from the post iPCR bead clean up.

18. Final HS Caliper QC

18.1. Use the 10X dilution plate on the Caliper GX according to following SOP:

LIBPR.0051 Operation and maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay

19. Pooling Samples into 1.5ml Tubes on Span-8 (if needed) or Rearray Unpooled Samples into 1.5ml Tubes

19.1. Refer to the following SOP for pooling on Span-8

LIBPR.0093 Span-8 Pooling of DNA Samples

20. Qubit QC on Pooled Samples or Unpooled Samples for submission

20.1. Refer to the following SOP for quantifying pooled samples:

LIBPR.0030 Quantifying DNA Samples using the Qubit Fluorometer

21. Sequencing Submission:

21.1. For each library, determine the corrected final molar concentration for submission to sequencing. Use the average base pair size previously obtained from the Caliper HS DNA profile and the result from the Quant-iT or Qubit to obtain the final size-corrected nM quant. The remaining final volume (after QCs) is ~20 µL.

Appendix A: LIMS Protocol

1. Start of Plate Library Construction– IDX pipeline
2. Bioanalyzer Run – QC Category: sonication QC
3. A-Bisulfite Library Construction - IDX pipeline
4. Plate_Indexed_PCR – IDX pipeline
5. Plate_PPBC_SizeSelection – IDX pipeline
6. Bioanalyzer Run/Caliper QC Category: Post library construction size selection QC.

   Enter the following attributes:
   - Library_size_distribution_bp (From Agilent or Caliper)
   - Avg_DNA_bp_size (From Agilent or Caliper)
   - DNA_concentration_ng_uL (From Quant-iT or Qubit)
7. If Pooling: Action: Aliquot pooling volume into a new TRA
8. If Pooling: Pooling and/or Manual Rearray into tubes – IPE pipeline
9. Final_Submission – PET (no pooling); IPE (pooled)

Appendix B: Bisufite Sequencing QCs

Post Shearing Profiles on Agilent HS DNA chip

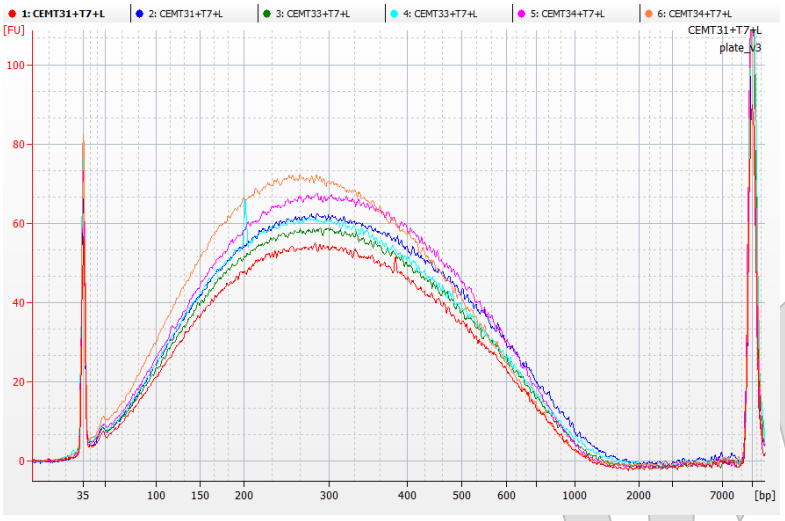


Fig 1. CEMT gDNA samples with spike in DNA post LE220 plate tube shearing (2*50s). Library profiles centre around 300bp on HSDNA chip. Libraries were diluted 6X prior to QC.

Final Library Profiles on Agilent DNA 1000 chip

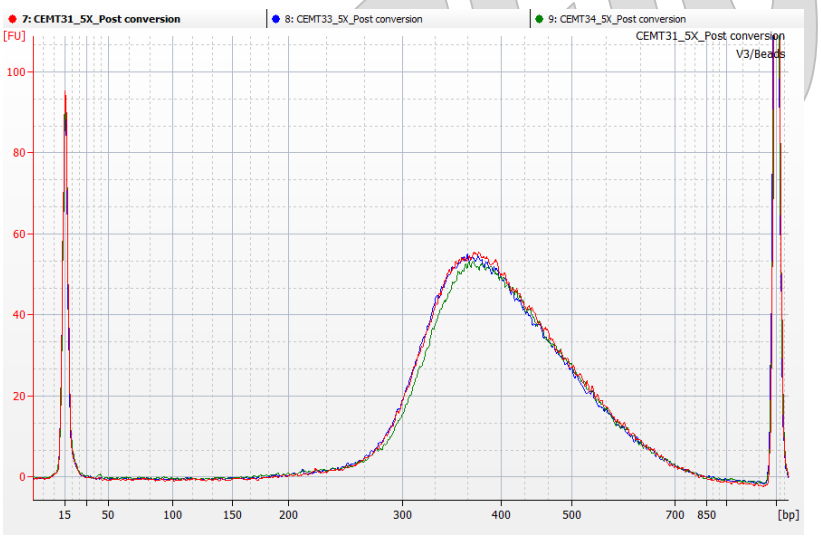


Fig 2. Final CEMT library profiles after bisulfite conversion and 5X cycles of enrichment using KAPA ura+ PCR enrichment kit. Library profiles peak ~375bp on DNA 1000 chip due to fragmentation during bisulfite conversion.

Appendix C
Bisulfite Conversion Clean up

Bead Binding
Transfer 150uL (all) of the converted DNA to 610uL of Bead Binding solution. Set a multichannel pipette to 160uL and mix 10X.

Conversion Mixture (µL)	Bead Binding Solution Volume (µL)	Mixing volume (µL)	# Mixes	Binding (min)	Magnet (min)	Supernatant Removal (uL)
150	610	160	10	15	7	760

Wash 1
Remove the plate from the magnet and add 133uL of M-Wash buffer to each well, mix 10X using a multichannel pipette. Repeat two times to a total of 399uL.

M-Wash Buffer (µL)	# Mixes	Binding (min)	Magnet (min)	Supernatant Removal (uL)	Dry time (min)
3x133uL	10 each	1	1	400	none

Desulphonization
Remove the plate from the magnet and add 200uL of desuphonization buffer, mix 10X using a multichannel pipette.

Desulphonization Buffer (µL)	# Mixes	Binding (min)	Magnet (min)	Supernatant Removal (uL)
200	10	15	5	200

Wash 2
Remove the plate from the magnet and add 133uL of M-Wash buffer, mix 10X using a multichannel pipette. Repeat 3X to a total of 399uL.

M-Wash Buffer (µL)*	# Mixes	Binding (min)	Magnet (min)	Supernatant Removal (uL)
3x133uL	10 each	1	1	400

Wash 3
Remove the plate from the magnet and add 200uL of M-Wash buffer, mix 10X

M-Wash Buffer (µL)	# Mixes	Binding (min)	Magnet (min)	Supernatant Removal (uL)	Dry time at 55C (min)
200uL	10	1	1	200	5

Final Elution Add pre-heated M-Elution buffer to dried beads, cover, quick spin and then incubate 4 minutes at 55C on the custom Peltier heater with 1.2mL adapter. Samples can be stored at -20C.

M-Elution buffer (uL)	Binding time at 55C (min)	Magnet time (min)	Transfer volume (µL)
25	4	2	24

Appendix D: Expert SOP: 96-well Bisulfite library construction

Step	SOP/Program name	Biomek protocol: LibraryConstruction>Bisulfite	LIMS protocols
1) Spike-in Lambda		> Spike-in Lambda	Start of plate library construction
2) Transfer DNA to Covaris plate		> Shearing Setup	
3) Shear DNA to 300 bp (LE220)	Plate_100sec_Bisulfite.elproc LIBPR.0097		
4) QC sheared DNA: QC 11/plate: Agilent HS DNA assay	LIBPR.0017		Bioanalyzer Run: QC type: sonication QC
5) Transfer out of covaris plate		> Transfer out of Covaris	
6) Bead clean sheared DNA		> Bead clean sheared DNA	
7) End Repair	ER (tetrad)	> End Repair	A-Bisulfite Library Construction
8) Clean up End Repair		> Bead Clean E. R.	
9) Adenylation	ATAIL_6040 (tetrad)	> A-Tailing	
10) 15 minute Ligation	20C_15 (tetrad)	> Adapter Ligation	
11) Adapter Clean up 2X, 1:1 Ligation clean up		> Bead Clean Ligation(2X)	
12) Bisulfite Conversion		> Bisulfite Conversion	
13) Clean Up Conversion		> Clean Up Conversion	
14) Indexing PCR	BS-5X (tetrad)	> Index PCR	Plate_Index_PCR
15) Post PCR size selection 2X, 1:1 bead:sample clean up		> Bead Clean iPCR (2X)	Plate_PPBC_SizeSelection
16) Dilute Libraries for QCs		> Dilute for QC	
17) QC Final Libraries Quant-iT HSDNA Assay Caliper HSDNA assay	LIBPR.0108 LIBPR.0051	> Quant-iT (10X dil.)	Bioanalyzer /Caliper Run: QC type: Post library construction size selection QC
18) Option: Span-8 equal M pooling	LIBPR.0093	Biomek Span-8: Project > LibCore > Pool_Samples_from_AB1000_or_Axygen PCR 96FS_to_microfugeTubes17mm	Action: -Aliquot to create pooling (TRA) -Rearray function to track (IX pool)
19) Option: Quantify pool	LIBPR.0030		

Qubit HS DNA assay			
20) Submit libraries			Final_Submission

Solutions: *Methylated_Ligation_40pmol; Bisulfite_LibConst_iPCR_Brew*